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REVIEW

Molecular basis of transitivity in plant RNA silencing

Shruti Choudhary[1] · Sapna Thakur[1] · Pankaj Bhardwaj[1]

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Abstract
The discovery of small RNAs has offered exciting opportunities in manipulating gene expression. The non-coding RNAs cause target gene inactivation at the transcriptional, post-transcriptional or translational level. In addition to the default silencing approach, they provide another mode of gene regulation by transitivity. Here, gradual amplification in effector RNAs number allows regulation of genes other than the original target and causes the outspread of silencing from its origin to aid a robust response. Unlike the short-range cell-to-cell movement of silencing signal (through plasmodesmata), little is known of the mediators of systemic silencing (usually through phloem). Through the present review, we combine the reports available so far to better understand the characteristics of secondary silencing, factors involved, and summarize the instances where it has been employed in plants. Understanding the molecular mechanism behind transitivity has led to the designing of efficient transgenes for targeted gene inactivation, utilized in silencing of a multigene family, and in the field of functional genomics. Studies uncovering the origin of distinct secondary silencing pathways in plants have been exploited for developing artificial RNA silencing methods such as hairpin RNA, artificial microRNA, intrinsic direct repeat, inverted repeat, artificial trans-acting siRNA, phased siRNA, and virus-induced gene silencing. The techniques have facilitated the spread of traits such as pathogenic resistance or alter plant growth and development features. The mechanism of reprogramming in the silencing machinery and the consequent genetic manipulation through transitive RNA is still not completely understood and its exploitation in crop improvement programmes is still in a developing phase.

Keywords Transitivity · Small RNA · Secondary silencing · Transitive RNAi · Robust RNAi

Modes of smRNA-mediated control

Explorations into the genomic landscape of eukaryotes revealed that the coding gene sequences are disrupted by non-coding regions. Later it was identified that the non-coding loci play a major role in regulating the coding components itself. One of the regulating mechanism involves gene silencing by means of small RNAs (smRNAs). The non-coding RNAs (21–26-nt) induce gene silencing through homologous base pairing. Their synthesis begins with the processing of double-stranded RNA (dsRNA) precursors by a ribonuclease-III-like enzyme (DICER). The dsRNA precursor is either derived from an endogenous source (locus, transposable element, repeat region, etc.) or introduced from outside (transgene or virus). The resulting smRNAs can be trans-acting microRNA (miRNA), which emerge from single-stranded (ss) RNA precursor with a self-complementary hairpin or stem–loop structure. While the double-stranded (ds) RNA, *e.g.*; products of RNA-dependent RNA polymerase (RDR), smRNA-target duplex, derivatives of viral replication, transgenes, etc. are the source of cis-acting short interfering RNAs (siRNA). Depending on their source, siRNAs are transgenic, viral (vsiRNAs), trans-acting (tasiRNAs), heterochromatin-associated (hcsiRNA), or natural antisense (natsiRNA). Table 1 enlists a few features of miRNA and siRNA. Once synthesized, different effector smRNAs are loaded onto RNA-induced Silencing Complex (RISC), which in turn comprises of Argonaute (AGO) and Dicer-like (DCL) proteins. Following processing and loading stages, there is a series of steps that culminate into target inactivation either at the transcriptional, post-transcriptional or translational level. Both mi/siRNA can implement PTGS control via cleavage of the target mRNA. However, siRNA possess an extra feature of RNA-directed DNA methylation

✉ Pankaj Bhardwaj
pankajihbt@gmail.com; pankajbhardwaj@cup.edu.in

[1] Molecular Genetics Laboratory, Department of Plant Sciences, Central University of Punjab, City Campus, Mansa Road, Bathinda 151001, India

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Table 1 Comparison of characteristic features of short-interfering (siRNA) and micro-RNA (miRNA)

Feature	siRNA	miRNA
Origin	Exo/endogenous	Endogenous
Conformation	Double-stranded RNA	Single-stranded RNA with self-complementary foldback structure
Length (nucleotides)	21–24 nt	19–25 nt
Biogenetic sources	Repeat/transposable elements, endogenous cleavage fragments, viral transcripts, transgenes	Non-protein coding *MIR* genes or introns of long RNA
Target description	Genes from which they originate	Genes other than their precursor
Mode of action	Usually *in cis*	*trans*
mRNA cleavage	Capable	Capable
De-adenylation	Incapable	Capable
Post-transcriptional or translational repression	Capable	Capable
Transcriptional silencing	Capable	Incapable
DNA methylation	Capable	Incapable
Transitivity	Capable	Capable
Systemic response	Capable	Capable

(RdDM) and chromatin remodeling, which further highlights the possibility that siRNA-mediated silencing might share some unifying features with epigenetic modifications. Overall, it can be concluded that the effect of smRNAs is observed at various levels including mRNA stability, nucleic acid degradation, heterochromatin assembly, DNA/histone methylation, transcription, and translational repression. The mode of genetic surveillance through smRNAs goes by different names—RNA silencing/post-transcriptional gene silencing (PTGS) in plants, RNA interference (RNAi) in animals, and quelling in fungi. Although slight variations in the silencing mechanism have been witnessed in different organisms yet, the fundamentals have remained highly conserved. An understanding of diverse pathways of the control exercised by smRNA has enlightened various concepts of tissue or stage-specific gene expression regulation. Numerous reports have documented their contribution to distinct biological functions. Right from the cell or tissue-specific differentiation [1] to the stability, expression, and even evolution of the entire genome [2, 3], the smRNAs have been proved essential during plant growth and development [4], stress [5], or immune responses [6].

Other than the default silencing mechanism discussed above and referred henceforth as primary (1°), there exist two additional features of PTGS. First is the transitive RNAi whereby, RDR activity extends the smRNA-target complex beyond the original trigger-spot. This subset of siRNA is known as transitive or secondary (2°) siRNA and the process is called transitivity [7]. To exemplify, miRNA cleavage of the target creates a cluster of 21-nt 2° siRNAs, known as phasiRNAs. The products of the transitive event have been reported to augment silencing by increasing the effectiveness and scope of RNAi (Fig. 1). The second feature is a systemic response i.e., silencing travels from one tissue to the other and eventually to the whole plant. A few independent reports on *Nicotiana benthamiana,* as reviewed by Pyott and Molnar in 2015, summarized: (a) the sequence-specific viral resistance, beginning from the infection site, dispersed throughout the foliage; (b) transgene-induced systemic GFP silencing phenotype was observed in wild-type plantlets; (c) the wild-type scions displayed a reduced nitrate/nitrite reductase expression similar to the traits of chlorotic and silenced stocks; and (d) GFP silencing signal could cross a three-way graft [8]. The downstream spread of silencing was observed for the first time in transformed tomato from the 5′ untranslated region and was later supported by other reports [9–12]. smRNA itself or in the form of a long precursor has been observed to enter the cells via plasmodesmata either through diffusion or protrusions of the endoplasmic reticulum. This transport was evident from the presence of long and short RNAs in the phloem exudates [13]. The short-range travel over a few cells differs from the long-distance spreading of silencing between tissues/organs. It suggests that the associated signal might not be a single molecule and that its generation, spread, and perception may follow multiple silencing pathways [14]. While a short-range signal diffuses passively, the long-range silencing through transitivity occurs by amplifying the 1° signals. Earlier siRNAs were assumed as the only mobile effectors until the involvement of tasiRNAs or miRNAs came up [15–17].

Summarising the above events, it is concluded that RNA molecule functions as a messenger to establish a systemic gene regulatory event. Movement of the silencing signal is correlated to the transmission of sequence-specific resistance

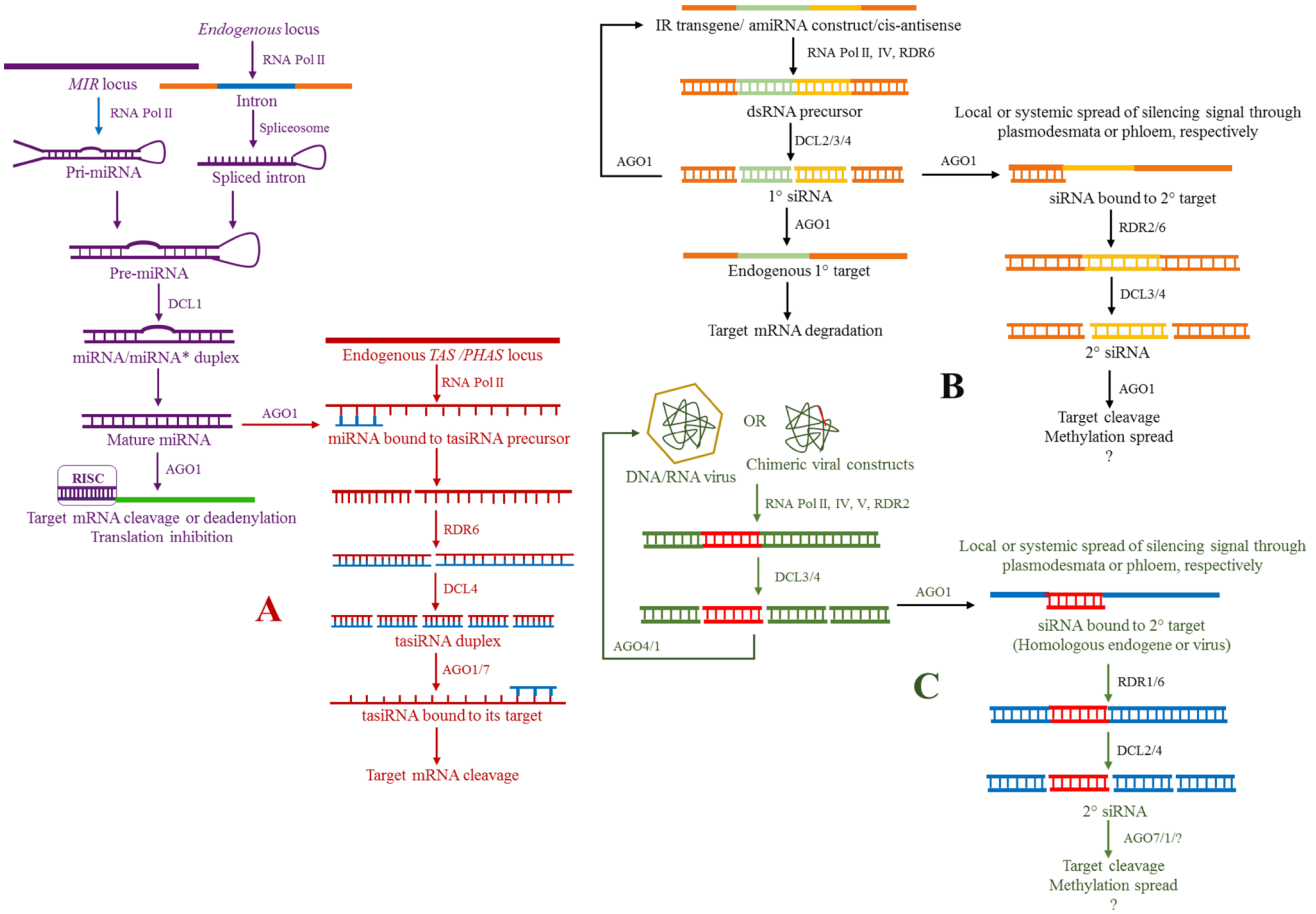

Fig. 1 A model of transitive or secondary RNA silencing in plants initiated from: **a** miRNA-mediated transitive production of tasiRNAs; **b** transgene or artificial miRNA constructs; **c** viral genome or chimeric constructs (? unknown role) (*Note* For the sake of simplicity, other interacting molecules/pathways are not shown)

from the site of virus infection to distant tissues; silencing in transgene-expressing plants, and transmission of PTGS from silenced rootstocks to non-silenced scions. Despite the fact that the content of transgene-derived mobile smRNAs is rather negligible in the recipient tissues, they are capable of producing readily detectable effects. Besides, the movement is more efficient from shoot to root strictly following the source–sink relationship [18]. It also explains the observations that, (i) wild-type roots accumulate ~ 100-fold more GFP-specific smRNAs than the non-transformed roots and (ii) GFP silencing initiated by grafting was first detected in lateral roots rather than in the vicinity of the graft-junction [19]. Briefly speaking, RNAi is a non-cell-autonomous event implying that it outspreads farther from the cell where the signal originates.

The present review focusses on the transitive or 2° responses of RNAi, the factors influencing its production, and finally their implications in plant systems. We will first explore the distinguishing features of a secondary mode of silencing that lead to spreading of regulation ranging both from short to local and systemic events. Since the advent of sequencing technologies, numerous types of 2° siRNA species have been identified in plants. Several experiments with smRNAs have indicated the extent of distant signaling and the nature of their non-autonomous effects. The mechanism for 2° siRNA biogenesis and their function, including the protein/non-protein components of both primary and dedicated transitive RNAi as well as the RNA turnover machinery will be discussed. The phenomenon of transitivity can have major consequences during genetic manipulations and various strategies are employed to increase the specificity, effectivity, and amplify the effect of silencing through transitivity. The factors associated with the transitive silencing reveal complex interactions among the molecular regulators, the mechanism of which is still largely unknown. However, recent studies with Arabidopsis, tobacco, tomato, and rice have advanced our understanding of silencing spread in plants. This review consolidates the reports to increase the understanding

of the transitivity triggering event of smRNAs, a poorly understood but relevant gene regulation phenomenon.

Characteristics of transitivity in plants

The smRNA-target complex similar to the one with primer-template is identified and extended by RDR. The duplex is then processed by DICER or DICER-like (DCL) homologs into 2° effectors. As the synthesis continues, there is a gradual spreading of the initial RNAi signal. The transitivity products control target stability, transcription, and translation similar to 1° smRNAs. Since the polymerase can proceed in one direction, transitivity is supposed to happen at a single end of the original target as seen in *Caenorhabditis elegans* [20]. On the contrary, the bidirectional spread of silencing, as testified by numerous reports in plants, is an anomaly [11, 21, 22]. As per a two-hit trigger model, siRNA synthesis occurs from the region within the two target sites [23] (Fig. 2). TAS3 precursors, having dual target sites for miRNA, laid the foundation of this model. *TAS1* loci involving double hits by miR173 and by master siRNA generated from TAS1c or TAS1/TAS2 led to the generation of 2° siRNAs. But the two phenomena, cleavage mediated by smRNA and transitivity, can be genetically uncoupled [22]. The 5′–3′ spreading explains the default de novo or primer-independent synthesis by plant RdRs. On the other hand, AGO1-bound siRNAs could serve as small bridges to lead RDR6 towards the 3′ end of the target. The binding of siRNA to a target, mimicking the template-primer annealing, act as a trigger for the synthesis of complementary RNA (cRNA) [24]. Additionally, the purified recombinant RDR2 and RDR6 demonstrated primer-independent as well as primer-dependent RNA polymerase activities in vitro but with different efficiencies [25]. Both RDRs can synthesize dsRNA either by extending the 21–24 nt RNAs bound to cRNA template or by extension of the self-primed RNA target. It was suggested that the RDRs had inherent feature for initiating the primed mode and that priming by RDR6 is more sensitive to in vitro conditions and/or the absence of cellular co-factors than RDR2. Overall the 2° synthesis activated by the binding of unique smRNA species is primed and unidirectional independent of an initial cleavage event (Fig. 2a). While, an aberrant RNA transcript, having multiple smRNA target sites, is responsible for unprimed, bidirectional, and cleavage-dependent transitivity (Fig. 2b). For instance, the aborted replication products during viral infections could trigger the unprimed transitivity.

Molecular factors behind transitivity

Precursor

The aberrant RNA model was put forward to define the precise molecular nature of the trigger. It states that a specific concentration of defective RNA is recognized by RDR6. The progenitors include (i) cleavage by-products, or (ii) unprotected RNA arising from polymerase processing errors, faulty RNA turnover/decay or antisense counterpart of a sense transgene [26, 27]. Later arose some exceptions that led to considering alternative models:

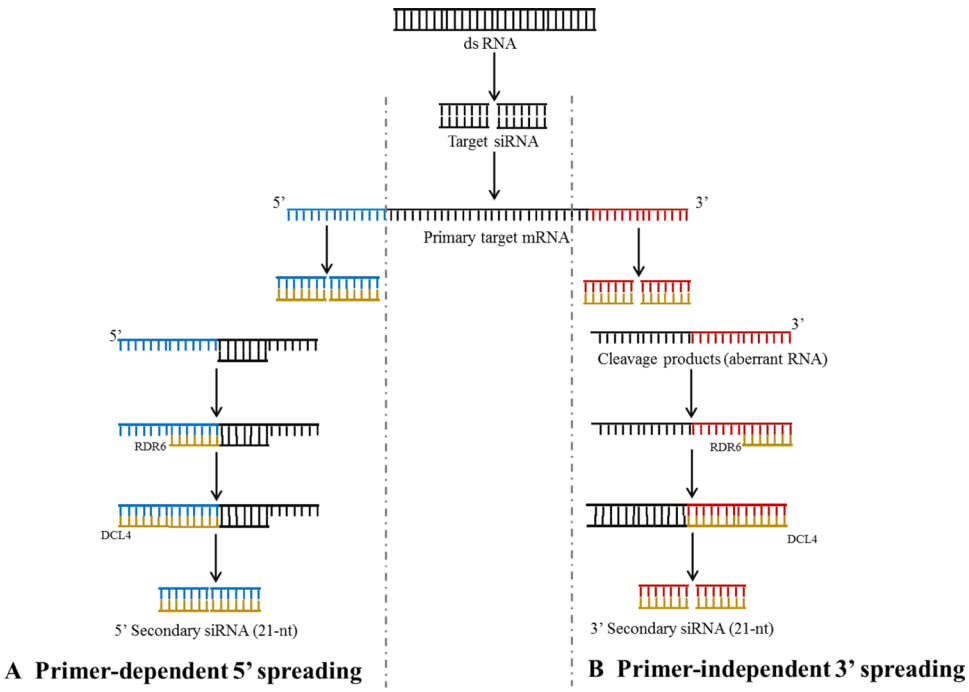

Fig. 2 In the transitive mode of silencing, a dsRNA source of 1° siRNAs promotes 2° siRNA production both 5′ and 3′ of the initial target. **a** Production of 5′ 2° siRNAs requires the RDR6-dependent complementary strand synthesis with 1° siRNA as primer and **b** for 3′ 2° siRNAs generation, RNA fragments arising from 1° cleavage, recognized as aberrant, initiate dsRNA synthesis. Pathways in **a**, **b** are not necessarily mutually exclusive, as 3′ 2° siRNAs produced in latter could further prime dsRNA synthesis according to the events depicted in **a**. Adapted from [24]

(i) 2° siRNAs mapped to the 3′-terminus of the cleavage site, (ii) 2° siRNAs were not detected in some mutants with 5′-cleavage fragments, and (iii) base pairing between miRNA and target sequences can predict the location of transitivity [28]. In this context, a RISC-trigger model stated that transitivity occurs when both 5′ and 3′ cleavage fragments are still together and bound to the miRNA. RISC is necessary to initiate transitivity and the 'dwell time of RISC on a target' will determine if transitivity will take place or not. A weak interaction of RISC with target allow its dissociation and follows RDR6 recruitment to either of the cleaved strands [29]. Nonetheless, the model failed in clarifying the case of RDR6-dependent siRNA production only in the double mutants of *xrn4*/*ski2* [30]. Two waves of transitivity—a combination of the above two models, was suggested as a solution to these exceptions [28]. Multiple rounds of siRNA amplification due to a defective RNA decay could result in stronger transitivity. At first, an initial trigger will produce low levels of 2° siRNA in the vicinity of the cleavage site. A sufficient degree of aberrant RNA transcripts further promotes their amplification to produce highly abundant siRNAs in the presence of RISC. However, the triggering can also be genetically independent of the amplification waves of transitivity.

dsRNA acts as the diffusible messenger during the progression of silencing. A silenced, transgene-containing rootstock could prompt PTGS when grafted to a non-silenced scion led to the discovery of mobile RNA signal [31–33]. The enhanced co-suppression (absent in non-silenced plants) displayed by smRNA complementary to both the strands of target indicated that dsRNAs are active participants in the spread of silencing. dsRNA may arise from endogenous loci, TEs, satellite regions, transgenes, any intron-containing transcript, and replicated- or stem-loop structures of the viral genome. 2° smRNA molecules produced from the slicing of dsRNA fragment act on the mRNAs that share sequence homology with the original trigger. In vivo cleavage of dsRNA introduced either directly or via a transgene/virus are well-studied examples of a transitive response. The cleaved by-products of silencing or the intermediates generated while the RNA turnover events can act as additional inputs generating more and more 2° effectors. A dsRNA precursor of 1° siRNA promotes 2° siRNA production in both 5′ and 3′ of the initially targeted region. 2° siRNA biogenesis in 5′-direction is clear from the RDR6-dependent cRNA synthesis primed by 1° siRNAs. In contrast, the 1° cleaved fragments act as aberrant transcripts to initiate dsRNA synthesis towards the 3′ end. Additionally, 5′ and 3′ biosynthesis may not necessarily be mutually exclusive as siRNAs produced in the latter case could trigger transitivity in a similar manner during 5′-transitivity. Other parameters such as the source of dsRNA (trigger); the nature and processing of original target; availability/non-availability of primer, proteins, co-factors or enzymes; and other dynamics of primary silencing, can also affect the occurrence and spread of transitivity.

miRNA

miRNA bound to one region of the target can sometimes induce 2° siRNA production from the entire length of the transcript. One such example is an endogenous target of miRNA, Arabidopsis *TAS* (*AtTAS*) loci. tasiRNA biogenesis is observed during the juvenile-to-adult transition and is a means to allocate smRNAs to the locations where they are at limiting concentration [34]. How an endogenous miRNA target like *TAS* loci enters the pathway of transitive silencing is unclear yet. An earlier study hypothesized that ss-tasiRNA precursors are cleaved by miRNAs and this cleavage site acts as a starting point for RDR6-catalyzed cRNA synthesis followed by phased DCL4 processing. 21-nt tasiRNAs are derived from the transcription of *TAS* genes by RNA Pol II and cleaves the *TAS* precursor RNA. RDR6 converts the 22-nt miRNA cleavage fragments of *TAS* transcript to long dsRNA, which is processed by DCL4 into siRNAs with 21-nt phasing from the cleavage site. Like miRNAs, tasiRNAs are methylated and interact with either AGO1/7 to degrade the target such as auxin response factors involved in a reproductive phase transition. Another speculation was that introns in the premature transcript could trigger transitive silencing. The miRNA-guided cleavage of an unspliced TAS1a transcript induced the production of intron-specific siRNA or tasiRNA that regulate 2° targets at pre-mRNA level [35, 36].

RISC-targeted 5′- and 3′-cleavage products left after the action of miRNA can also serve as a template during transitivity. RISC identifies a peculiar asymmetry, as in the structure of miRNA/miRNA*, which is determined both by the passenger as well as the guide strand and trigger transitivity [37]. In general, the phasiRNA biosynthesis exhibits the involvement of RISC. Numerous tasiRNA-like siRNAs, collectively called phased siRNAs or phasiRNAs with the arrangements ranging from 21-, 22-, and 24-nt, have been identified in plants. While 21-nt tasiRNAs are generally transcribed from the *TAS* intergenic loci and generated from the processing by RDR6-AGO1/7-DCL4, the phasiRNAs are similar to tasiRNAs but they have a broader regime of origins. The phasiRNAs can originate both from protein-coding and non-coding sequences such as transposable elements (TEs) and repetitive DNA. Overall, it can be concluded that the tasiRNA is a type of phasiRNA. The phasiRNAs derived from the nucleotide binding site-leucine-rich repeat (NBS-LRR) disease resistance genes regulate plant defense genes [38, 39]. Similarly, phasiRNAs originating from TEs identified in the vegetative nucleus of pollens, in vitro dedifferentiated cells, and in DNA methylation mutants prevent

heterochromatic or transcriptional silencing [40]. A recent report proposed that the origin of reproductive phasiRNAs might be evolutionarily linked to anther emergence [41]. Two pathways in monocots were demonstrated to yield abundant phasiRNAs: (i) miR2275 triggering 24-nt phasiRNA (parallel with meiosis) and (ii) 21-nt phasiRNAs in premeiotic anthers. The study also demonstrated a dynamic evolution of 24-nt phasiRNA pathway with its wide occurrence in eudicots, while being absent in legumes and Arabidopsis. In addition to 24-nt phasiRNAs in Solanaceae species (but not through the miR2275 trigger), some loci displayed 12-nt phasing also, which suggested the phasiRNA implications in microspore development [41]. Another form of miRNA (22-nt species) directed against TEs can promote 2° smRNA biogenesis [42]. Cell-specific expression of miRNAs is tightly regulated and so is the transitivity. For instance, the assembly of RISC can be modified by manipulating the expression of *MIR168* locus and its target gene, *AGO1,* and, therefore, control the abundance of other miRNAs [43]. In some cases, an alternate site for miRNA binding might be present on the target, which enables only siRNA production, irrespective of the fact that the target is cleaved or not. The evidence came from a study where individual mutations induced within the target were shown to prevent only the miRNA-directed degradation but not transitivity [44].

siRNA

siRNA arise from the perfect dsRNA structures of the transcripts derived from endogenes, transgenes, viral genomes, and even miRNA targets. siRNAs can cross plasmodesmata to nearby cells and move further to remote tissues via phloem during transitive gene silencing. The phloem sap isolated from silenced lines and viral-infected plant tissues was analyzed to identify the smRNA species involved in systemic signaling [45]. It confirmed the presence of transgene-specific siRNA and vsiRNA in the vascular bundles. Furthermore, a phloem smRNA-binding protein was found necessary in the trafficking of 25-nt ssRNA form. A recent model of transitive silencing clarified that the siRNA-target complex can be identified as a template for the production of 2° siRNA to target mRNAs irrespective of its homology to the first inducer [46].

siRNA subsets are classified by their generation and the role they play in transitive silencing. Entry of viruses in plants triggers the production of vsiRNA or viral induced siRNA. The dsRNA replication intermediates or 2° structures in the viral genome generate vsiRNAs through RDR and DCL2/4 processing [47]. During this antiviral defense in plants, these effectors can either cause endonucleolytic cleavage or DNA methylation of the viral transcripts [48]. Repeat or transposon-associated siRNAs are responsible for 2° RdDM inducing epigenetic changes during transcriptional (TGS) and post-transcriptional gene silencing (PTGS). dsRNA from natural antisense loci transcribed by RNA Pol II can be cleaved by RDR2–DCL2 into natsiRNA, a subset mediating PTGS of homologous sequences during stress responses [5]. siRNAs are induced not only through inherent dsRNAs but also from the factors present outside the host genome such as transgenes introduced either through direct or vector-mediated transformation approach. Similarly, aberrant transcripts of external origins or those generated during the host's RNA quality control process can give rise to 2° siRNA. In addition to these, the hcsiRNA loci are considered to evolve into novel miRNA genes that can shed light on the origin of 2° siRNA biogenesis [49]. However, further evaluations are required to establish a relationship between 2° siRNA, miRNA, and 23–24-nt hcsiRNA.

RDR, AGO, and DCL

Sequence-independent RDR synthesizes cRNA from a template and is an indispensable component of the transitive RNAi machinery [50]. The enzyme can spot even a small amount of trigger and convert it to dsRNA to generate 2° siRNAs. RDRs were once thought to mediate only transgene-induced silencing (TIGS) but later came their precise cellular implications in TGS and PTGS pathways. The functional discovery of RDRs was made possible through the smRNA deep-sequencing [51–54]. The entry of invading pathogens, and transgenes and TEs could induce plant RDR2 and RDR6. RDR6, further amplifies the antiviral response, the scarcity of which can increase the plant's susceptibility to these infections. In addition, an efficient RNA silencing during development and during hairpin RNA (hpRNA) or sense/IR-mediated TIGS also depend upon RDR6 [5, 55–57]. Similarly, RDR2 is also connected with genome stability and heterochromatin formation [58, 59].

AGO family proteins, central to PTGS, are recruited to specific target through complementary base pairing guided by si/miRNA. In addition to the target degradation, the slicer activity of AGO has been implicated in siRNA loading and tasiRNA biogenesis [60–62]. AGO is involved not only in the phasing of tasiRNA, but it is held that "direct recruitment of RDRs via AGO proteins underlies 2° siRNA production in animals, plants, and fungi" [62]. AGO1, which otherwise bond with 21-nt species only, can be reprogrammed to identify 22-nt sized species during processing and loading of the asymmetric miRNA/miRNA* duplexes. Hence, AGO1 could interact with both 21- and 22-nt miRNAs by different mechanisms to engage the RDR6-associated amplification apparatus towards transitive siRNA production from mRNA and tasiRNA [42]. Similarly, AGO4-loaded 24-nt siRNA acts as mobile silencing signal to induce epigenetic changes. ago4 mutants displayed (i) lesser epigenetic modifications at certain chromosomal loci, (ii) a downregulated

accumulation of 24-nt siRNAs, and (iii) hypomethylation at several repeats, transposons, and retrotransposons [63]. Other than AGO1, AGO2 and AGO7 loaded with smRNAs are also predisposed to trigger transitive silencing.

The third major protein involved with the transitive RNA processing is DCL of the ribonuclease III family. Generally speaking, DCL1 yields 21- or 22-nt miRNA; DCL2 generates 22-nt siRNA from ds viral genome/transgene; DCL3 processes for 24-nt hcsiRNA; and DCL4 is mainly involved with the 21-nt siRNA, including tasiRNA [64]. In the context of transitivity, DCL4 primarily favors the biogenesis of transitive smRNA. DCL1-dependent miRNA triggers tasiRNA synthesis via DCL4. Nuclear DCL4 produces 21-nt tasiRNAs from non-coding TAS transcripts and other siRNA species from a transgene. In addition to DCL4, DCL3b (a paralog of DCL3) protein in rice is required for the biogenesis of 21/24-nt phasiRNA (Table 2). Further, it is observed that DCL2 can also process the products of RDR6 and although less studied, it can cause transitive silencing of transgene transcripts. Overall, it was stated that hierarchical redundant activities of both DCL2 and DCL4 are necessary for transitivity [22, 27].

Protein interactions

DCL, AGO, and polymerase are the fundamentals in the siRNA biogenesis as well as in the RNAi. As observed in the model plant, a different polymerase (RDR) works with a specific combination of ribonuclease (DCL1–4) and splicer (AGO1–10) to produce distinct 2° siRNA species in vivo (Table 2). RDR6–DCL4/2–AGO1 can directly deliver siRNA or similar species from aberrant or even functional transcripts originating either from a virus, transgene, endogenous TAS or TAS-like locus, or 3′/5′-cleavage product. The multi-domain DCL converts the dsRNA into 21/23-nt siRNA, cleaves the 70-nt precursor RNA stem-loop structures into 21–23 nt miRNA, and leads to the 5′/3′ 2° siRNA biogenesis. The miRNA–AGO1 complex attacks the TAS transcript, a non-coding precursor transcribed by RNA Pol II. RDR6 converts the cleaved TAS transcript into dsRNA, which is diced by DCL4 into 21-nt 2° siRNAs that display a unique 'phased' or 21-nt long spaced pattern. Similarly, intracellular and long-range silencing triggered by inverted repeat (IR) transgenes or virus is correlated with RDR6 and accumulation of 21-nt siRNAs [65].

During viral infections, RDR1 (induced by salicylic acid signaling) produces vsiRNA that show sequence homology with the host's genes [57, 66]. smRNAs of the distinct size range are detected in plants infected with RNA viruses of different families and DNA geminiviruses. DCL4/2-dependent 21 and 22-nt vsiRNAs are mainly implicated in RNA–VIGS; DCL2 and DCL3 in 22 and 24-nt geminiviral siRNAs, respectively, and an additional DCL activity can also generate 21-nt geminiviral siRNAs. According to a model for antiviral defense, it is proposed that viral dsRNA is processed primarily by DCL4 assisted by DRB4 and secondarily by DCL2 to further enhance the multiplication of siRNAs. RDRs simultaneously regulate both siRNA biogenesis and a systemic antiviral silencing. The replication/transcription products of the virus display the characteristics of an unprotected or aberrant RNA and serve as templates for RDR1 or RDR2 during the amplification in antiviral silencing [67]. RDR6 is also involved in systemic transgene silencing via 21-nt 2° siRNAs and promotes the immune response of plant against viruses.

In addition to 21-nt siRNAs, the RdDM-associated 24-nt siRNAs are also referred to as mobile signals. In a transgene meristem silencing system, 1° RdDM

Table 2 A summary of various protein classes involved in the generation of different length small RNA species

1° smRNA species	Precursor	DCL	AGO	RDR	Accessory	Transitivity	References
21/22-nt miRNA	Stem-loop (*MIR* genes, introns of endogenes)	1	7/1	–	RNA Pol II, HYL1, SERRATE, HEN1	Yes	[42, 73, 74]
21/22-nt siRNA	dsRNA (IR of endo/transgene, virus)	2/4	1	6	RNA Pol II, SGS-3, DRB4	Yes	[22, 27, 64]
24-nt siRNA	dsRNA (transgene, endogene)	3	1	2	RNA Pol IV/V, HEN1, DRD1	Yes	[63, 64]
24-nt hcsiRNA	dsRNA (transposon, heterochromatic, 5S ribosomal genes, inverted and direct repeats of endo/transgenes)	3	4	2	RNA Pol IV, HEN1, DRM1/2, DRD1, DRB?	Not clear	[62–64]
24-nt natsiRNA	*cis*-Antisense gene pair	2	?	6	RNA Pol IV, SGS3, HEN1, DRB?	Yes	[5, 70]
21-nt natsiRNA	*cis*-Antisense gene pair or natsiRNA precursor	1	?	6	HEN1, SGS3, DRB?, DCL2 processed 24-nt natsiRNA	–	[5, 71]
21-nt tasiRNA	dsRNA (derived from non-coding TAS loci)	4	1/7	6	SGS3, DRB4, HEN1, HYL1, SDE5; RNA Pol II; DCL1 processed miRNA-directed cleavage	Yes	[37, 42–44]
21/22/24-nt vsiRNA	dsRNA (virus)	4	1	1	SDE3, SGS3; Salicylic acid	Yes	[57, 66, 67]

attracts the 2° siRNA-synthesizing machinery (RNA Pol IV–RDR2–DCL3) resulting in the spread of methylation upstream the regulatory regions [68]. Eamens et al. and Daxinger et al. further specified that methylation can be uncoupled from transitive RNAi events as it requires factors other than RDRs i.e., RNA pol IV and V [68, 69]. Methylation spread has also been reported from endogenous loci, but the production of associated effectors require DRD1 and RNA Pol V-mediated DNA methylation. The combination of DCL2, RDR6, SGS3, and DNA-dependent RNA Pol-like subunit NRPD1a are behind the production of 24-nt cis-antisense siRNA [70]. The resulting siRNA-guided cleavage of one of the two parent transcripts can trigger the production of a series of 2° phased 21-nt natsiRNA. The function of this 2° natsiRNA is currently unclear. The role of 1° natsiRNA in adaptive responses and the widespread antisense transcription observed in plant genomes reflect a comprehensive gene regulation [71].

Apart from the 'RDR–DCL–AGO' trio, numerous other proteins and co-factors interact with one another during RNA transitivity (Table 3). The first one is the helicase activity of Silencing Defective 3 (SDE3) employed during the silencing amplification in Arabidopsis [72]. Second is the DRBs like HYL1 and SERRATE, work with DCL1 to predominantly process 21-nt miRNAs from the foldback precursors loaded onto AGO1. Similarly, DRB4 and DCL4 function together to process RDR6-dependent dsRNA precursors for biosynthesis of tasiRNAs also associated with AGO1. Next in line are the accessory proteins like SGS3, co-localized with RDR6 in cytosolic siRNA-bodies, can associate with AGO1 to induce tasiRNA formation. It can prevent the degradation of TAS transcripts by keeping them away from RNA decay factors and act to stabilize the cleavage fragments [73, 74]. Also, VIGS triggered by both RNA virus (RNA–VIGS) and DNA vectors (DNA–VIGS) require RDR6 and its cofactor SGS3 [75]. Fourth protein is methyltransferase, which can also directly or indirectly influence siRNA multiplication. Methylation of the 3′-terminal nucleotide at the 2′-OH position by HEN1 methyltransferase protects all plant smRNAs from degradation and oligouridylation [76]. RdDM at cytosine residue by the RDR2/DCL3-processed 24-nt siRNAs (derived from endogenous loci) is directed by AGO4/Pol V complexes and another methyltransferase, DRM2 (DOMAINS-REARRANGED METHYLTRANSFERASE2). Similarly, S-adenosylhomocysteine hydrolase is required for sustaining methylation and can enhance local and systemic silencing by an RDR6-dependent increase of 2° siRNA [77]. Lastly, RNA quality control and turnover systems keep a check on the undesired indulgence of the silencing machinery. XRN exonucleases and nonsense-mediated decay can destroy the cleavage fragments produced from the siRNA-loaded AGO1 complex [78] and miRNA targets [79], respectively. Elucidating the mechanism underlying coordination of these accessory factors to channelize the amplification, beginning from the template recycling, can shed more light on the regulation of transitivity.

Characteristics of the primary target

The nature of the 1° target affects gene suppression through transitivity. Transgenes are comparatively more prone to

Table 3 A summary of protein factors involved in distinct small RNA pathways as observed from the reports available in plants

Protein	Function
DCL1	natsiRNA, miRNA, phasiRNA synthesis; ribonuclease; dsRNA binding activity; helicase; negatively regulate DCL3/4 [73–75]
DCL2	22-/24-nt siRNA, natsiRNA synthesis [5]; antiviral defense [47]; transitive silencing of transgenes; stress resistance in egg cells [119]
DCL3a	24 nt hcsiRNA synthesis via processing of Pol IV & Pol V products and DNA/chromatin methylation [68, 69, 83, 84]
DCL3b	24-nt phasiRNA synthesis; stamen-specific 24-nt phasiRNA production [1, 98, 99]
DCL4	Synthesis of 21-nt phasiRNA, 21-nt siRNA, tasiRNA, 2° siRNA [64]
AGO1	RNA slicer in TAS1abc/2–miR172 and TAS4–miR828 pathway [15, 94, 95]; mi/miRNA interaction; sense-mediated PTGS [83, 84]; tasiRNA synthesis from 3′ cleavage fragments [37]
AGO4	RdDM [77]; interacts with long miRNAs and hcsiRNAs [85]
AGO5	Inhibits megagametogenesis in somatic cells; regulates cell division of premeiotic germ cells [119]
AGO2	Mediates ds break repair [120]
AGO7	RNA slicer in TAS3–miR390 pathway [97]; tasiRNA synthesis from 5′-cleavage transcripts [63]
HEN1	3′ methylation of smRNAs [76]; all siRNA pathways [72, 73]
RDR6	ssRNA into dsRNA [24]; endogenous 21/24-nt nt siRNA production [42, 88]; antiviral defence [68]; transitivity [45]
RDR2	ssRNA into dsRNAs that are substrates for DCLs [5]; endogenous 24-nt siRNA production [25, 58, 59]; antiviral defence [67]
SGS3	Antiviral defence [75]; endogenous 21/24-nt nt siRNA production [70]; transitivity [71–74]
DRM2	De novo DNA methylation [76, 77]
DRB4	Interacts with DCL4 to generate phasiRNA [67]

the RDR6-mediated transitivity than the endogenes. One of the suggestion in this context was that the endogenous mRNAs are either uniquely marked or are less abundant which makes them more immune towards RDR. Due to the fact that transgenes express more as they are coupled with strong promoters, it was concluded that they exhibit transitivity more frequently. However, the concentration parameter does not always hold true as transitive siRNAs were neither detected in the transgenic plants overexpressing *VIRP1* [80] nor against the most abundant endogene, *RUBISCO* [12, 81]. Similarly, most of the miRNA targets, except a few like *TAS* loci, resist 2° amplification by RDR6 [53].

There is no clear justification to clarify this trend of transitivity and several logics put forward in this context. Binding of proteins to exon–exon junctions, intron splicing, and other mRNA processing events can interfere with RNAi and thus, transitivity. A group of researchers are of the view that mRNA maturation includes protected termini, 3′-polyadenylation and 5′-methylated G-capping, can be a means of safeguarding endogenous transcripts from the attack by RNAi [82]. The transgene-derived transcripts lack 5′/3′-protection and apparently, the mRNA-associated factors such as cap/tail binding proteins and exon junction complexes. As a result, the naked mRNAs tend to adopt bulged asymmetric or hairpin conformations and hence, become vulnerable to RDR-directed pathways. This also describes the RDR2/6–DCL4–AGO1/4-arbitrated initiation of transitivity from the dsRNA arising from viruses or RNA pol IV substrates (TEs/repetitive DNA elements), both of which are structurally aberrant in terms of folding, capping, and faulty termination [83, 84]. Supporting the hypothesis, it was observed that the mRNA decay pathways, which destroy the aberrant or cleavage products prevent transitive silencing [28]. xrn4 mutants of 5′–3′ exoribonuclease display a higher abundance of RISC 3′-cleavage fragments and are more susceptible to RDR6-mediated TIGS [82] as well as produce siRNA from many endogenous loci [85]. Additionally, the intron sequences when integrated to 1° target can sustain transitivity not only in a transgene with multiple introns but along endogenes as well [86]. Explaining the transitivity originating from the endogenous loci such as *TAS*, the tasiRNAs were demonstrated to arise from the regions within the intron.

Miscellaneous factors controlling transitivity

The 2° amplification of the smRNA population depends on time, while the magnitude of sequence similarity between the trigger-target duo ensures efficient silencing. The capability of smRNA molecules to inactivate a target for a specified duration of time is defined as the efficiency of RNAi. A high degree of homology between the trigger and 1° target increase the effectiveness and incidence of transitive suppression. Initially, a lag phase is seen during transitivity that corresponds to the time required by RDR to reach 1° target and build up a steady-state level of 2° siRNAs before attaining a plateau maxima. The abundance or length of the trigger also affects transitive competence. To demonstrate, the hairpin structure of dsRNA formed from the folding of a longer sequence will be far less stable than those arising from a shorter one. Although increasing the substrate length would increase the number of resultant siRNA molecules, but in such cases, transitivity will take a longer time to establish. The long cDNA insert when placed between the original target and the region of homology with the 2° target will reduce the spread of transitivity. An 'XYZ' system to silence both transgenes and endogenes in *Arabidopsis thaliana* was adopted by Bleys et al. [46]. They demonstrated that a larger distance between the direct target (X) and adjacent sequences (Z) in the chimeric construct (Y) could postpone transitivity. Increasing the intron length in the premature mRNA delayed the onset and had deleterious effects on the progression and degree of silencing in 2° target [86]. The level, spread, and efficiency of the transitive RNAi further depend upon the amount of 2° siRNAs produced. Likewise, the extent of homology between 1° target and endogene has a direct and positive impact on the frequency, efficiency, degree, and strength of suppression. The production of transitive siRNAs from the coupling of trigger and target can, therefore, be considered as a function of the distance, length, and time.

Inferences in the plant system

The mobility of smRNA is necessary to establish a gradient of target mRNA during plant development (top to bottom leaf patterning and vascular bundle formation), relieving stress (during phosphate starvation), and directing an epigenetic control. Similarly, the exogenous ones like vsiRNA can move around locally and promote a systemic antiviral response in uninfected cells. These instances present the way that the mobile silencing can be exploited in crop improvement. Transitivity has been exploited to varying extents in plants ranging from maintenance of homeostasis to providing control during stress. The smRNA-target duplex in the presence of RDR and other co-factors provoke a cascade of reactions producing 2° smRNA that in turn provide a robust silencing effect. The level of 2° siRNAs is directly proportional to the degree of silencing movement. A dispersal of RNA silencing over short-range of a few cells can occur through symplastic movements and over a longer range throughout the plant through recurring short-distance signaling events. The event concludes in three phases: (a) signal (dsRNA) intake, (b) signal intensification and dissemination to other cells, and (c) target suppression. Its occurrence

was demonstrated to be persistent through multiple developmental stages, even capable of passing to the progeny and through grafts. However, not all the processes might necessarily involve the transitivity as the DCL-mediated processing of dsRNA might give rise to distinct smRNA species. Thus, the potential of different smRNA species to induce local, transitive or systemic silencing may vary. In this section, we elaborate the transgene or virus-based technologies developed to induce RNA silencing in plants. These technologies are also widely used in functional studies and genetic engineering for plant improvement.

Regulating the multi-gene family

The strategic increase of the silencing efficiency offers numerous advantages: (a) smaller amounts of dsRNA can induce the response throughout the body, (b) spread of silencing regardless the mode of dsRNA introduction, (c) target gene selection from whole of the genome, and (d) study of genes in life stages where other methods of dsRNA delivery is not possible. Firstly, the amplification of silencing related RNAs suggests that an active, persistent silencing could be initiated with small amounts of initiator dsRNA. An advantage of transitive siRNAs lies in elucidating the gene function by knocking down the test gene(s), controlling gene expression, and in understanding the effects of silencing on cell function and organism development. Earlier approaches that involved generating knockout mutants or tagged lines for each of the single gene of a multigene family can be cumbersome and time-consuming. However, if cDNA dataset is available, single/multiple gene-specific suppression can be achieved using 3′UTR or highly conserved gene sequences as a substitute. Similarly, transitivity can allow for the smRNA-mediated regulation of multigene families by using a single gene construct designed from a highly conserved region. Even the expression of genes not belonging to the same family can be suppressed using this approach if they share certain conserved regions. Several methods are available to test natural and engineered constructs in plants and their effect on the movement of silencing. The *OsRac* family in rice comprises of seven different genes each of which possesses a highly conserved coding region that exhibits high sequence homology. Miki et al. demonstrated that multiple genes could be suppressed by a single IR construct using a diverged 3′UTR as the trigger [81]. Likewise, 2° siRNA accumulation was observed in both stable and transient transformants expressing *OsSLR1* [87]. It was further suggested that transitivity could lead to knock-down of the endogenes that share sequence homology outside the sequence of *OsSLR1* gene-specific tag. Thus, a highly specific sequence is necessary to suppress only one member of a gene family.

Inverted–repeat (IR) transgene

The presence of IR within a transgene results in a complete fold-back dsRNA structure and is a frequently employed approach in gene 'knock-down' providing an elevated and efficient response [88]. The *I* locus, an IR cluster, facilitated down-regulation of the *CHALCONE SYNTHASE* (*CHS*) genes (*CHS7/8*), which leads to yellow seed coat due to anthocyanin deficiency. dsRNA, generated from the 27-kb region of the *I* locus, has clusters of *CHS1-3-4* and *CHS4-3-1* genes that are processed into 1° siRNAs. Amplification by RDR further generates 2° siRNA, which can silence all members of the *CHS* family [87]. smRNA sequencing traces the amplification of 1° to 2° siRNAs during seed development stages (post-fertilization to maturity) in soybean [89]. Amplification of 2° CHS siRNAs at a premature developmental stage concluded that the regulation of the *CHS* genomic region occurs very early during seed development. Younger seeds had a higher proportion of 1° 22-nt CHS siRNAs that increased with the development. The 22-nt siRNAs, though in lesser abundance, were enough to trigger the amplification of 2° 21-nt CHS siRNAs from the target. Silencing occurred only in the seed coat which further recommended that the 1° siRNA levels might be insufficient to trigger transitive amplification in other tissues [89]. Another transgene structure, comprising of a sense target gene sequence followed by an IR of the *nopaline synthase* (*nos*) terminator, was utilized in tomato and Arabidopsis [90]. For this construct, a target sequence, not homologous to the *nos* gene, was cloned in front of the nos IR and arranged in an antisense-sense manner separated with a spacer. Here, tasiRNA-like 2° siRNAs were suggested to be induced by the downstream IR structure. Both antisense *nos* sequence and sense 5′*nos* sequence were transcribed to form a hpRNA structure and later, processed to yield siRNAs, which in turn target 3′ region of the fusion transcript to trigger 3′ to 5′ transitivity [90].

Two strawberry endogenes, *CHS* & *O-METHYLTRANSFERASE,* were compared for their silencing efficiencies as an inducer and transitive vector system [91]. Each of the designed vectors contained an inverted hairpin construct, both sense–antisense nucleotide sequence of a fragment separated by an intron. All the sequence fragments demonstrated a significant downregulation of gene expression along the entire length of both the targets. Deep sequencing detected 2° siRNA hotspots within < 200-nt region and transitivity outside the 1° region, suggesting a propagation of the silencing signal. However, the endogene regulation was less effective by the transitive method as 1° siRNA were far high in number than the 2° siRNA. A transgenic target could also induce a transitive spread of methylation. 2° signal originating from a 1° transgenic target simultaneously silenced 2° transgene as well as *CATALASE* endogene [92].

Although both endogene and transgene displayed transitive silencing but their susceptibility of experiencing 2° RdDM varied as only the latter could exhibit transitivity-mediated methylation.

tasiRNA/phasiRNA-mediated genetic manipulation

miRNA-triggered 2° siRNA generation from protein-coding genes has been illustrated in various plants [38, 39, 53, 93–96] and mostly identified based on the phasing patterns as in tasiRNAs and phasiRNAs. miR390 descendants including miR7122, miR1509, fve-PPRtri1/2, and miR173 target a subgroup of ~ 40 pentatricopeptide repeat (PPR) containing gene superfamily to generate phasiRNAs [97]. Similarly, 2° siRNA production through the NBS–LRR superfamily initiate from miR482/2118 that target the P-loop motif sequence in the mRNA for disease resistance proteins [38]. miR828 and miR858 target ~ 81 transcripts of MYB family to trigger phasiRNAs production [94, 95]. The resulting 2° effectors further target distinct genes and thus, expand the miRNA-mediated regulatory network. In the same way, the young developing panicles of some plants were found enriched with phasiRNAs clusters [1, 98, 99]. Rice genome encodes for long non-coding RNAs that are targeted by 22-nt long miR2118 and miR2275, which in turn produce 21- and 24-nt phasiRNAs, respectively. High expression of both the miRNA triggers, miR2118/2275, is found in rice and maize stamens. It is speculated that *OsDCL3b* evolved a panicle-specific expression pattern to produce stamen-specific 24-nt 2° siRNAs or phasiRNAs. Such studies can provide further insights into the mechanisms of tissue specificity regulation by smRNA and evolution of the loci generating phasiRNAs. Likewise, *TAS3* and *TAS4*, non-protein-coding loci, produce 2° siRNAs [51, 61]. Degradome sequencing from soybean detected *TAS* genes as the source of 2° siRNAs [100]. Another locus to discuss here is the F-box receptors of the TIR1/AFB2 Auxin Receptor (TAAR) family that controls auxin signaling. miR393 regulates the expression of all the four members of the TAAR family, which in turn triggers the formation of functional 2° siRNAs, known as siTAARs. siTAARs might play some role in miR393-associated responses as the mir393b-1 mutants showed impaired biogenesis of miR393b as well as siTAAR and mild developmental defects [101].

Methodologies based on phasiRNA synthesis that employ amiRNA transgene constructs have been used in gene silencing. A vector is designed by replacing the endogenous miRNA/miRNA* sequence with the desired amiRNA/amiRNA* sequence. The desired amiRNA will accumulate after transcription resulting in the silencing of endogenous targets and can simultaneously knock down multiple genes by triggering the production of 2° siRNA [37, 102, 103]. A variant of this method is miRNA-induced gene silencing (MIGS) that was demonstrated in Arabidopsis. It involved fusing a target gene upstream the miR173 target site (miR173ts) to trigger the production of phasiRNAs downstream of the cleavage site [104]. An efficient silencing in case of *CHS* and *PDS* (phytoene desaturase) was also displayed in case of the transformed petunia. Through deep sequencing, it was concluded that the processing of miRNA precursor in petunia differs from that in Arabidopsis. miR173ts cleaved as expected while an abundant 2° siRNAs accumulated from multiple sites of endogenous *CHS-A* and *CHS-J* regions. They projected that the ability of miR173-induced siRNA to initiate transitivity is similar to that siRNA functioning in co-suppression and hpRNA silencing [105]. Further with a simple vector coupled to transitive amplification of signals from endogenous transcripts, MIGS can be a good silencing approach, especially for regulating a cluster of homologous genes with similar functions.

Artificial tasiRNA (atasiRNA) is another technique, which bypasses off-targeting as is the case with MIGS [106–108]. The miR173-targeted TAS1 transcript modified to express artificial atasiRNAs effectively induces silencing of selected endogenous genes [83, 84]. Here, the sequence fused to an upstream miR173ts is altered by replacing the native 21-nt native phased siRNA sequences within *TAS* transcript with an antisense target gene sequence of the same length [109]. In addition, constructs which retain the miR173ts and TAS1 sequence is replaced with a relatively long target sequence can also induce effective silencing. On the contrary, substituting miR173ts with the sequences of the 21-nt miR167 or miR171 counters the silencing effect of the atasiRNAs. Like amiRNA constructs, atasiRNA constructs are relatively convenient to prepare than the hpRNA constructs. tasiRNAs can also induce DNA methylation at the corresponding *TAS* loci implying the potential of atasiRNA transgenes in subjecting RdDM-mediated transcriptional self-silencing [110]. Artificially engineering a cleavable 5′ miR390 target site allowed tasiRNA production from the original *TAS3* target and it was further observed that the 2° siRNA production can be triggered without cleavage [111].

Another silencing method uses a complementary 22-nt amiRNA sequence, which also imparts the benefits of mobile smRNA. Silencing of *CHS* gene was induced in the leaf using conventional hpRNA and 21 and 22-nt amiRNA plant expression vectors with Arabidopsis RUBISCO small subunit promoter [103]. In contrast to the hpRNA and 21-nt amiRNA that could trigger *CHS* silencing only in leaf, the 22-nt amiRNA induced silencing in all the tissues including roots and seed coats. This spreading of silencing was invoked by the RDR6-mediated accumulation of phasiRNAs from the endogenous transcript. These results established the efficacy of 22-nt amiRNA over hpRNA and conventional amiRNA in allowing the phasiRNA generation and its activity in mediating widespread silencing. The technology can

also assist in suppressing an entire gene family. Because many plants are polyploid or might have faced partial polyploidization at some stage of evolution, gene duplication or functional redundancy can be a problem in functional analysis. In such cases, all the genes of a cluster are knocked down by phasiRNA to determine an overall function of the cluster and then, a targeted amiRNA is used to identify the individual gene function. Although the phasiRNA-based approaches have some advantages over other silencing tools, yet they are not well investigated in plants.

Gene silencing through virus-mediated transitivity

Silencing a plant endogene by infecting with a virus containing sequence or an IR fragment of that gene gives a dynamic response. The host produces dsRNA in response to the entry of pathogens/parasites and after initiation of the event, RDR amplifies the original RNA signal to > 20 fold [67]. Pathogen-activated siRNAs (say, vsiRNAs) spread through the vascular system of the infected plant to generate an antiviral defense in non-affected cells via a transitive cascade. vsiRNA, which share sequence homology with host RNA, trigger 2° siRNA from the region adjacent to the target sequence. The mobile smRNA signaling favored the resistance against Prunus necrotic ringspot virus to reach in wild-type sweet cherry scions and against tobamoviruses in non-transgenic tobacco shoot stock [112, 113]. Deep sequencing and degradome analyses concluded that only a small fraction of vsiRNAs could degrade viral RNA and have an inefficient targeting effect. On the other hand, amplified vsiRNAs can transcend through the phloem to the whole plant establishing a systemic immunity [56, 114]. 2° siRNA derived from specific miRNA targets are also significant against filamentous pathogens [115–118]. Thus, amplification or systemic silencing acts in immune defense to relocate RNAi in distant tissues where the infection has not been spread yet.

It has been found that a plant gene sequence, when inserted into a virus, will become a target for degradation and leads to VIGS of the homologous plant mRNAs also. This forms the basis of vector designed for a VIGS system whereby a viral cDNA construct incorporates the host-derived target sequences. This construct will trigger the silencing of the homologous gene at both post-transcriptional and transcriptional levels. DNA virus is well-suited for studying virus-induced RNA silencing because their replication encompasses cytoplasmic and nuclear stages in the host. Furthermore, the DNA viruses do not encode their RDRs and hence, are also useful for examining the role of the host's RDRs in different silencing pathways. Secondly, the transfer of RNA between pathogens and plants is a two-way portal, i.e.; the pathogens can counter attack the plant defense in response to their survival. Many viruses and bacteria can bypass the silencing machinery of the plant and hinder the synthesis or movement of the systemic signal. Characterization of viral suppressors of RNA silencing (VSR) established that these suppressors target various components of the host's silencing mechanism. Transgenic plants expressing various VSRs were designed to see the effect of VIGS amplification and transitive RNAi at endogenous and transgenic loci [22]. Despite a strong interaction between VSRs and plant's siRNA that restricted the RDR6-mediated up/downstream amplification of silencing, the efficacy of transitivity was not affected as such. Minute quantities of 21-nt 1° siRNA, but not 24-nt siRNA, were able to trigger efficient transitivity. The 3′–5′ transitivity progressed over > 750-nt and after that decreased towards 5′-proximity. Target cleavage by 1° smRNA supported in vivo priming in this particular reaction and can be genetically uncoupled from unidirectional transitivity. Similarly, the helper component-proteinase of Potato virus Y and coat protein of Tomato chlorosis virus interacts with individual components of host silencing machinery to suppress silencing but instead, promoted the spread of systemic silencing [77]. The RNA silencing suppressors from fungal pathogens can also interfere with the accumulation of 2° siRNA to promote infection suggesting that the transitive RNAi pathway is necessary for plant immunity [116]. In the host-induced gene silencing, transfer of endogenous plant smRNAs can keep a check on pathogen gene expression. The successful silencing of targeted genes in pathogens using smRNA constructs indicates that plants may produce anti-pathogen smRNAs as a defense mechanism. Modulating host silencing pathways can alter the virulence of filamentous eukaryotic pathogens. A future prospect in this area is to characterize more silencing suppressors from other pathogens. Identifying the host targets of these suppressors will facilitate in understanding the eukaryotic pathogenesis in order to enhance resistance to plant diseases.

Summary

The small non-coding RNAs derived from double-stranded RNA destroy their target mRNA, which shows sequence homology with the inducer; the basic concept of gene regulation via silencing. High throughput sequencing and whole genome tiling array transcriptome made possible the functional discovery of non-conserved small RNAs in phylogenetically-distant plant species and unraveled the gene regulatory networks to a great deal. Genes encoding for the core components of silencing have diversified their role in higher organisms to play multiple roles in development and physiology, abiotic/biotic stress signaling, and evading foreign particles. An amplified and a mobile signal facilitating the silencing spread outside the inducer region is recognized in the form of transitive production of siRNA. A transition

of sequence-specific 1° siRNAs to 2° siRNAs targeting the regions outside the original target sequence in both 5′ and 3′ of the initial interval as observed in plants reflects primer-dependent/independent RDR6 activities. The components of RNAi machinery, their compartmentalization, interactions, transport, and dependency on other factors, will all determine the nature and extent of siRNA amplification. Endogenous TAS loci, pathogens, TE loci, transgenic or artificial smRNA constructs designed for specific as well as for multiple targeting, can induce local/systemic spread of silencing. Though several DCLs and RDRs are reported to produce distinctive sizes of 2° siRNA species, their role in mediating transitive still remains to be identified completely. The transitive event favors a spatiotemporal control over gene expression and regulation of an entire gene family through the use of tissue-specific promoters or by designing chimera having a certain homologous sequence of multiple genes of the same family. A blend of transitivity and 'target mimicry' can further unveil plant agronomic traits. However, there should be a clear distinction between different species of small RNA in mediating transitivity-dependent or independent spread of silencing. Therefore, crafting the 2° silencing experiments necessitates the careful selection of the source of RNAi signal, trigger or dsRNA, and 1° transcript. Furthermore, several genes regulated by small RNAs, especially 2° siRNAs, involved in plant–pathogen interactions and other cellular processes are still at large. Identifying these components would reveal the mechanism by which transitivity can affect these interactions and play any role in physiology and development of plants.

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Author contributions PB conceived and designed the research work. SC collected the data and wrote the draft. ST further discussed and improved the review. All authors have read and approved the final manuscript.

Compliance with ethical standards

Conflict of interest The authors declare no competing interests

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